# Reconstitution of photosynthetic oxygen evolving activity by rebinding of 33 kDa protein to $CaCl_2$-extracted PS II particles

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Received 24 November 1983

On extracting the $O_2$-evolving Photosystem II (PS II) particles with $CaCl_2$ solution, three proteins of 33, 24 and 16 kDa were completely liberated from the particles concomitant with total inactivation of $O_2$ evolution, but most of the Mn atoms present in the initial particles were preserved unaffected being still associated with the particles. On reading the extracted proteins to the extracted particles, a considerable amount of 33 kDa protein rebound to the particles concomitant with significant reconstitution of $O_2$-evolving activity.

*PS II particle* *Reconstitution* *Mn-binding* *$O_2$-evolution* *33-kDa protein*

## 1. INTRODUCTION

Despite a considerable amount of kinetic information available including the basic scheme for linear 4-step oxidation of water [1, 2], the biochemical mechanism of water oxidation to evolve $O_2$ is still far from understood. It is only recently that 3 proteins with approximate molecular masses of 32, 24 and 16 kDa were recognized to be closely related to the function of photosynthetic $O_2$ evolution.

The above 3 proteins have been shown to be easily liberated by various treatments. On monovalent salt-extraction, 24 and 16 kDa proteins are specifically liberated from the particles concomitant with partial inactivation of $O_2$-evolving activity, while leaving Mn and 33 kDa protein associated with the particles [3,4]. The liberated proteins are capable of rebinding to the extracted particles concomitant with appreciable enhancement of $O_2$ evolution [3]. This enhancement has sometimes been called reconstitution, but it does not always seem to be reconstitution in the true sense, since the inactivation induced by monovalent salt extraction is not complete, i.e. by about 50% and 80% in terms of DCIP photoreduction with water as donor and $O_2$ with DMQ as acceptor, respectively ([3,4] see also table 1). In fact, it is still a matter of argument whether 24 kDa protein plays an essential [5] or regulatory [6] role in $O_2$ evolution.

On Tris- and/or alkaline-extraction, all of the above 3 proteins are liberated from the particles concomitant with release of Mn, and $O_2$ evolution is totally lost [7–9]. During extraction, release of Mn proceeds in parallel with liberation of 33 kDa protein [4,10]. This observation has been taken as an implication for the close involvement of this protein in maintaining Mn on membranes. In spite of many indirect indications about the role of this protein, no successful reconstitution of $O_2$-evolving activity has so far been reported with the particles depleted of this protein. Probably, the simultaneous release of Mn and 33 kDa protein has made reconstitution experiments difficult.

Under these circumstances, a technique for discrete liberation of 33 kDa protein leaving Mn unaffected has been desired. Quite recently, we found that divalent salt-extraction completely

*Abbreviations:* Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DMQ, 2,5-dimethylbenzoquinone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS II, Photosystem II

*Published by Elsevier Science Publishers B.V.*
001457593/84/$3.00 

liberates all the 3 proteins and completely inactivates $O_2$ evolution, whereas almost all of the Mn is preserved safely in the particles after removal of these proteins [11]. We report here a successful reconstitution by using such divalent salt-extracted PS II particles.

## 2. EXPERIMENTAL

The $O_2$-evolving PS II particles were obtained from spinach as in [12] with the modification in [11]. The particles were washed 3 times before use to remove nonspecifically releasing material. The particles were then extracted with 1 M $CaCl_2$, 300 mM sorbitol, 10 mM NaCl and 40 mM Mes-NaOH (pH 6.5), and centrifuged (35 000 × $g$, 15 min) to precipitate $CaCl_2$-extracted particles. The resulting supernatant was concentrated with polyethylene glycol, PEG 2000. The $CaCl_2$-extracted particles were resuspended (250 $\mu$g Chl/ml) with 300 mM sorbitol, 10 mM NaCl, 10 mM $CaCl_2$ and 40 mM Mes-NaOH (pH 6.5), and subjected to reconstitution by addition of various amounts of concentrated $CaCl_2$-extract. After incubation in darkness at 0°C for 1 h, the reconstituted particles were pelleted, washed once, and then suspended in 300 mM sorbitol, 10 mM NaCl, 40 mM Mes-NaOH (pH 6.5). Determinations of $O_2$ evolution (polarographically), DCIP photoreduction (photometrically) and protein composition (SDS-PAGE) and Mn abundance (atomic absorption) were done for the particles before and after reconstitution with initial PS II particles as reference.

## 3. RESULTS AND DISCUSSION

Fig. 1 shows the activity of $O_2$ evolution before and after reconstitution. The high activity (320 $\mu$mol $O_2$/mg Chl per h) of initial particles (a) was completely abolished by $CaCl_2$-extraction (b), but the activity was significantly restored after reconstitution (c), and the restored activity was sensitive to diuron. The activities before and after reconstitution were 2 and 28%, respectively, of the original activity of initial particles. Similar reconstitution was confirmed in terms of DCIP photoreduction (table 1).

In fig. 2, the protein compositions of the particles before and after reconstitution are shown. In

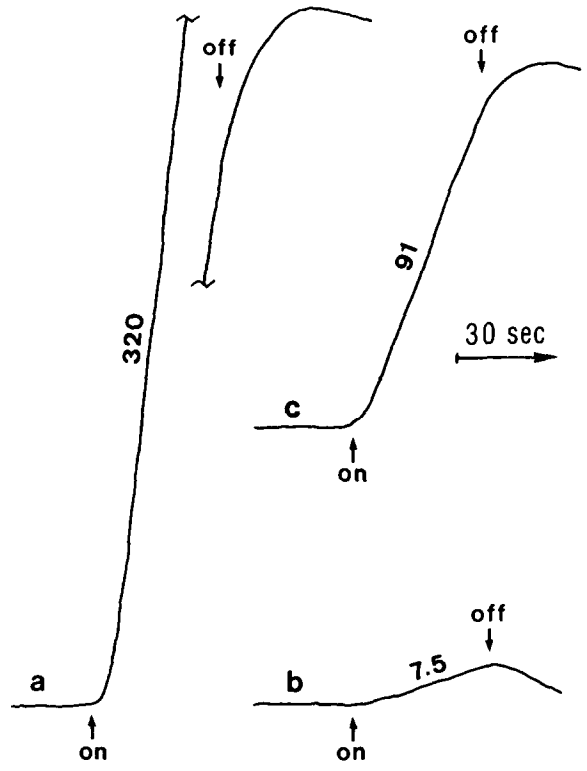


Fig.1. Course of $O_2$ evolution in reconstituted PS II particles: (a) control particles, (b) $CaCl_2$-extracted particles, (c) reconstituted particles. Figures on each trace are activities in $\mu$mol $O_2$/mg Chl per h.

$CaCl_2$-extracted particles, the 3 proteins of 33, 24 and 16 kDa were totally absent (b), but after reconstitution, a significant amount of 33 kDa protein was found in the particles, although the other two proteins were not (c). It is of note that the present reconstitution involves only 33 kDa protein but not 24 and 16 kDa proteins, so that the activity restoration observed here is a phenomenon obviously different from the activity enhancement due to rebinding of 24 kDa protein as reported in [3] or [13].

In table 1 the abundance of Mn in the particles before and after reconstitution is shown. In sharp contrast to Tris-extraction but similar to NaCl-extraction, $CaCl_2$-extraction did not liberate any Mn from the particles and almost all the Mn atoms present in initial particles were found in extracted particles even after total liberation of 33 kDa protein. The Mn abundance was also not greatly changed during reconstitution, and more than

Table 1

Mn abundance and water oxidation activity in terms of $O_2$ evolution and DCIP photoreduction in salt-extracted and reconstituted PS II particles

| PS II particles | Water oxidation activity ($e^-$ $\mu$equiv./mg Chl per h) | | Mn abundance (atoms/400 Chl) |
|---|---|---|---|
| | $H_2O \rightarrow DMQ$ | $H_2O \rightarrow DCIP$ | |
| Control | 1280 (100%) | 734 (100%) | 10 |
| 1 M $CaCl_2$-extracted | 30 (2%) | 50 (7%) | 9.2 |
| 1 M $CaCl_2$-extracted and reconstituted | 364 (28%) | 430 (59%) | 9.0 |
| 1 M NaCl-extracted | 248 (19%) | 396 (54%) | 9.9 |
| 0.8 M Tris-extracted[a] | – | – | 0.2 |

[a] Data in a separate experiment

90% of the Mn present in extracted particles were found in reconstituted particles.

The above results indicate that on liberation of 33 kDa protein, the Mn atoms lose their function even if they remain associated with the particles, but recover their function on rebinding of the protein to extracted particles. This indication was further confirmed by the relationship between activity regeneration and the amount of 33 kDa protein rebound to particles. As shown in fig. 3, the activity regeneration increased with increasing amount of $CaCl^2$-extract readded to extracted particles, and the extent of activity regeneration plotted against the amount of 33 kDa protein found in reconstituted particles gave a straight line as shown in the inset. The linear relationship shown here indicates clearly that the activity reconstitution is caused solely by rebinding of 33 kDa protein to extracted particles.

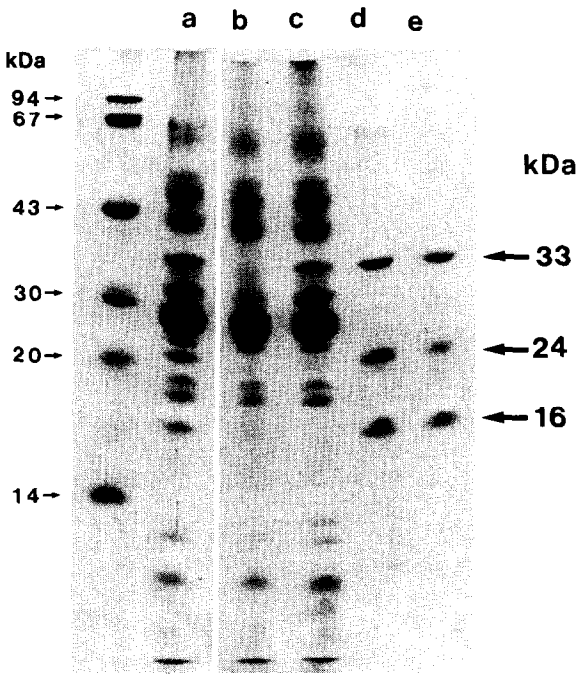


Fig.2. SDS–PAGE profile of PS II particles (left lane, marker proteins): (a) control particles, (b) $CaCl_2$-extracted particles, (c) $CaCl_2$-extracted and reconstituted particles, (d) 0.8 M Tris (pH 8.3)-extract, (e) 1 M $CaCl_2$-extract.

The maximal extent of activity reconstitution in terms of $O_2$ evolution was only 28% of the original activity in initial particles. One may think this extent is not large enough. When, however, we take into account that liberation of 24 kDa protein by NaCl-extraction results in 80 and 50% loss of $O_2$ evolution and DCIP photoreduction, respectively (see table 1), the present value is rationally considered significant, since the reconstituted particles in this study do not contain any 24 kDa protein. It is of note that both activities of $O_2$ evolution and DCIP photoreduction in the reconstituted particles exceed those in NaCl-extracted particles. The reason for the failure in reconstituting 24 kDa protein may be the presence of $CaCl_2$ during reconstitution (0.01–0.1 M), which was inevitably brought into the medium together with the $CaCl_2$-extract used as a source of extracted proteins. We have confirmed in a separate experiment

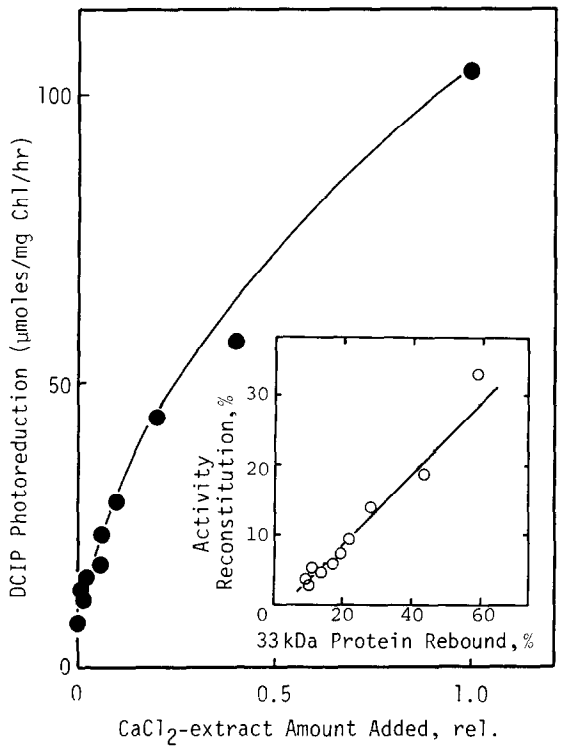


Fig.3. Dependence of reconstitution extent on the amount of $CaCl_2$-extract readded to $CaCl_2$-extracted particles. Inset: relationship between the extent of activity reconstitution and the amount of 33 kDa protein rebound to the particles. The amount of 33 kDa protein rebound was estimated from the peak area on SDS-PAGE densitogram of the protein relative to that of PS II reaction center protein (43 plus 47 kDa).

that $CaCl_2$-extraction at 0.1 M liberates both 24 and 16 kDa proteins but not 33 kDa proteins. If we reduce by some means that $CaCl_2$ concentration in the reconstitution medium, a higher extent of activity reconstitution will be achieved concomitant with rebinding of not only the 33 kDa but also the 24 kDa protein.

The present study revealed that the Mn atoms associated with PS II particles lose their function in water oxidation when 33 kDa protein is liberated from the particles, and restores their function when the protein rebinds back to the particles. The results are the first success in direct reconstitution of $O_2$-evolving activity, and confirm that:

(i) the Mn atoms are not bound to 33 kDa proteins but are probably bound to either or both of the 47 and 43 kDa proteins which constitute the PS II reaction center [14]; and

(ii) the role of 33 kDa protein is not to bind Mn to the particles but to maintain the conformation of the Mn atoms, probably 4 Mn/reaction center, on this (these) protein(s).

The $CaCl_2$-extracted PS II particles reported here will be highly advantageous in elucidating the molecular architecture of the $O_2$-evolving system of photosynthesis.

## ACKNOWLEDGEMENTS

This study was supported by a research grant on Solar Energy Conversion by Means of Photosynthesis given by the Science and Technology Agency of Japan (STA) to The Institute of Physical and Chemical Research (RIKEN).

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